
(12) UK Patent Application (19) GB (11) 2 114 978 A

- (21) Application No 8236905
(22) Date of filing 30 Dec 1982
(30) Priority data
(31) 3954
(32) 28 Dec 1981
(33) Hungary (HU)
(43) Application published
1 Sep 1983
(51) INT CL³
C07H 15/22 17/04
(52) Domestic classification
C2C 1672 167X 214 215
221 225 22Y 253 25Y
28X 306 30Y 321 322
32Y 340 34Y 360 361 362
364 36Y 509 50Y 610 627
634 640 643 650 652 660
662 670 672 70Y 801 KB
LH PN VA
U1S 1311 C2C
(56) Documents cited
None
(58) Field of search
C2C
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(54) Microbiological process for
preparing nebramycin-2,
nebramycin-5' and nebramycin-4

(57) Fermentation broths containing
either practically solely nebramycin-5'
(6''-O-carbamoyl-tobramycin) or a
mixture of nebramycin-2 (apramycin)
and nebramycin-5' at any desired
preset ratio, and nebramycin-4 (6''-O-
carbamoyl-kanamycin B), in which
a.) for preparing fermentation broths
containing practically solely
nebramycin-5' the MNG 204 strain of
Streptomyces tenebrarius, or
b.) for preparing fermentation broths
which contain nebramycin-2 and
nebramycin-5' at any desired preset

ratio the spores and vegetative
mycelia of any nebramycin complex
producing Streptomyces tenebrarius,
preferably the MNG 169 strain, and
those of any Streptomyces
tenebrarius, producing practically
solely nebramycin-5', preferably the
MNG 204 strain, are mixed at the
required ratio, and cultivated in a
medium containing organic carbon
and nitrogen sources, inorganic salts,
trace elements, oils and/or fats of
animal and/or plant origin in a
submerged, aerated culture, at 33 to
40°C, preferably at 37°C.

The antibiotic(s) can be isolated
from the fermentation broths by
simple procedures known per se.

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[illegible]

Fig. 1

SPECIFICATION

Microbiological process for preparing nebramycin-2, nebramycin-5' and nebramycin-4

The invention relates to a microbiological process for preparing fermentation broths which contain either practically solely nebramycin-5' or a mixture of nebramycin-2 and nebramycin-5' at any desired preset ratio and nebramycin-4.

It is known that nebramycin, a complex mixture of several antibiotics, is a biosynthetic metabolite of *Streptomyces tenebrarius* (Stark, Hoehn and Knox, *Antimicrobial Agents and Chemotherapy*, 1967, p. 314; British Patent No. 1,178,489 and U.S. Patent No. 3,691,279). The antibiotic mixture consists beyond several minor factors of the following major components: nebramycin-2 (apramycin), nebramycin-4 (6''-O-carbamoyl-kanamycin B), and nebramycin-5' (6''-O-carbamoyl-tobramycin) (Koch, Davis and Rhoades, *J. Antibiotics* 26, 745, 1973). In U.S. Patent No. 3,853,709 a microorganism producing nebramycin-7 in addition to nebramycin-2 is described. Japanese authors in U.S. Patent No. 4,032,404 are coproducing nebramycin-2 and nebramycin-5' by cultivating *Streptoalloteichus hindustanus*. According to Hungarian Patent No. 176,103 *Streptomyces tenebrarius* MNG 169 and 170, cultivated at submerged, aerated conditions, produce fermentation broths containing either nebramycin-2, nebramycin-5' and nebramycin-4 or nebramycin-2 and nebramycin-5', resp. These antibiotics are isolated according to Hungarian Patent No. 174,315.

Nebramycin-2 inhibits the growth of phytopathogenic and zoopathogenic microorganisms (U.S. Patents No. 3,691,279, 3,853,709 and 3,876,763), while both kanamycin B, obtained by hydrolyzing nebramycin-4, and tobramycin (Brulamycin), obtained by the hydrolysis of nebramycin-5', are commercial, broad-spectrum antibiotics playing a major role in human therapy in the treatment of infections caused by polyresistant bacteria (e.g. Preston and Wick, *Antimicrobial Agents and Chemotherapy* 1970, p. 322).

Applying the process of Hungarian Patent No. 176,103 an antibiotic mixture of the following composition is obtained (Table 1):

TABLE 1

Strain	Nebramycin-2	Nebramycin-4 percent	Nebramycin-5'
MNG 169 ⁺	77	2	21
MNG 170 ⁺⁺	91	traces	9

⁺ Deposited on December 29, 1980 at the National Collection of Microorganisms, National Institute for Public Health, Budapest, and available to the public since February 28, 1980.

⁺⁺ Deposited on April 5, 1978 at the same depository; available to the public since February 28, 1980.

It is apparent that nebramycin-2 is the major product of biosynthesis in both strains while nebramycin-5' has to be separated from the other predominant components by a highly sophisticated and expensive procedure.

The composition of an antibiotic complex, biosynthesized by a microorganism, is determined primarily by the cellular genetic information pool, and only to a much smaller extent by the fermentation conditions.

In the processes known up till now, the component ratio in the fermentation broth cannot be altered according to actual commercial requirements. Furthermore, if the production of nebramycin-5' is required, it has to be separated from the predominant nebramycin-2 by highly expensive procedures. In such multicomponent systems the evaluation of strain-selection experiments which aim to increase strain productivity is rather labour-intensive and time consuming. Following thin-layer chromatographic separation each component is quantified separately by a biological assay. It would be advantageous to carry out these strain selections with a one-component system enabling simple assay procedures. Furthermore, the increase of productivity in a strain biosynthesizing a single antibiotic, is more promising, since in this case the cells can utilize their entire biosynthetic capacity for forming a single compound.

It is the aim of this invention to develop a new and simple fermentation process for preparing fermentation broths which contain either solely nebramycin-5', or a mixture of nebramycin-2 and nebramycin-5' at any desired preset ratio.

In the course of our experiments it was unexpectedly found that *Streptomyces tenebrarius* MNG 169, treated with ethidium bromide (2,7-diamino-10-ethyl-phenyl-phenanthridinium bromide), furnishes a mutant strain which fails to produce nebramycin-2 but produces nebramycin-5' as a major

component beside traces of nebramycin-4. This phenomenon is rather unexpected and surprising as up till now ethidium bromide has not been applied as a mutagenic agent at all.

For the isolation of a mutant biosynthesizing an antibiotic complex with an altered component ratio, the spores of *Streptomyces tenebrarius* MNG 169 were cultivated in a liquid nutritive medium. The grown culture was divided into five parts, and 0, 0.05, 0.5, 5.0 and 50.0 $\mu\text{g/ml}$ ethidium bromide was added. Cultivation was continued for 24 hours, then one portion of the culture was spread on agar plates following dilution, while the other portion was used to inoculate liquid media containing equal amounts of ethidium bromide, and their cultivation continued for a further 24 hours. Then the above plating and inoculating procedure was repeated. The new liquid cultures obtained were repeatedly plated after 24 hours, the agar plates were incubated, some separate colonies were isolated and their productivity was investigated according to a procedure described in the Examples.

From the several hundred isolates originating from *Streptomyces tenebrarius* MNG 169 two mutant strains failed to produce nebramycin-2, but produced relatively small amounts of nebramycin-5' and traces of nebramycin-4. To improve the productivity of these two mutants producing nebramycin-5', strain selection and medium optimization experiments were carried out. In the course of these one mutant was lost while the production capacity of the second one became increased fivefold and could be maintained at this level. This strain was deposited on September 2, 1981 at the National Collection of Microorganisms (National Institute for Public Health, Budapest, Hungary) where it has been assigned accession No. MNG 204.

Furthermore the invention relates to a process for preparing fermentation broths containing a mixture of nebramycin-2 and nebramycin-5' at any desired preset ratio by mixing spores or vegetative mycelia of the original *Streptomyces tenebrarius* MNG 169 strain and those of the new *Streptomyces tenebrarius* MNG 204 strain, not producing nebramycin-2, at a required ratio and subsequently cultivating them in a mixed culture. Surprisingly, in the course of this fermentation performed with a mixed culture both strains produce higher levels of the antibiotic than in fermentations where each strain is cultivated separately.

Based on the above, the invention relates to a process for preparing fermentation broths which contain either practically solely nebramycin-5' or a mixture of nebramycin-2 and nebramycin-5' at any desired preset ratio and nebramycin-4. According to the process of the invention

- a) for preparing fermentation broths containing practically solely nebramycin-5' the MNG 204 strain of *Streptomyces tenebrarius*, or
- b) for preparing fermentation broths which contain nebramycin-2 and nebramycin-5' at any desired preset ratio and nebramycin-4 the spores or vegetative mycelia of any nebramycin complex producing *Streptomyces tenebrarius*, preferably the MNG 169 strain, and those of any *Streptomyces tenebrarius* producing practically solely nebramycin-5', preferably the MNG 204 strain, are mixed at the required ratio, and cultivated in a medium containing organic carbon and nitrogen sources, inorganic salts, trace elements, oils and/or fats of animal and/or plant origin in a submerged, aerated culture at 33 to 40°C, preferably at 37°C.

According to a preferred process of the invention the production of fermentation broths containing nebramycin-5' beside traces of nebramycin-4 is carried out by cultivating *Streptomyces tenebrarius* MNG 204 or one of its mutants, variants or recombinants. In the course of the fermentation 10 to 180 $\mu\text{g/ml}$ of nebramycin-4 is accumulated in the fermentation broth beside 2100 to 2600 $\mu\text{g/ml}$ of nebramycin-5', depending on cultivation conditions. Nebramycin-5' is isolated from the fermentation broth and separated from nebramycin-4 and various impurities by a single chromatographic step, then it is hydrolyzed to tobramycin by methods known per se. Eventually nebramycin-5' can be converted into tobramycin already in the culture and isolated therefrom.

According to a further preferred process of the invention for producing fermentation broths containing beside nebramycin-4 a mixture of nebramycin-2 and nebramycin-5' at any desired preset ratio, the spores and vegetative mycelia of *Streptomyces tenebrarius* MNG 169 and those of *Streptomyces tenebrarius* MNG 204 are mixed at the required ratio, and cultivated in a mixed culture. The antibiotics produced are isolated according to methods known per se, preferably according to Hungarian Patent No. 174,315. The ratio of the amount of nebramycin-2 and nebramycin-5' biosynthesized is nearly proportional to the cell number of the *Streptomyces tenebrarius* MNG 169 and MNG 204 strains, mixed at identical phases of growth.

It is obvious to any expert having ordinary skill in the art to which said subject matter pertains that the above deliberations are only valid in fermentation systems where the mixed cells are proliferating at nearly identical rates and where fermentation conditions are favourable to the biosynthesis of both components. Otherwise the ratio may be shifted in either direction. For an expert the fixing of the mixture ratio can not pose any problem at all.

According to the present invention the microorganisms are cultivated in a nutritive medium containing an organic carbon source, e.g. starch, glycerol, fructose or other similar compounds, preferably glucose; an organic nitrogen source, e.g. nutmeal, corn-steep liquor, peptone, yeast extract or other similar preparations, preferably soymeal, or casein, amino acids, preferably glutamic acid; an inorganic nitrogen source, preferably ammonium chloride, ammonium nitrate and ammonium sulfate; inorganic salts, preferably magnesium sulfate, zinc sulfate, cobalt nitrate and calcium carbonate; animal

- and/or plant fats and/or oils, e.g. palm oil, linseed oil, sunflower oil, rapeseed oil, pig fat or others, preferably a 1:1 mixture of palm oil and linseed oil; furthermore trace elements essential for the growth of the microorganism, at submerged, aerated fermentation conditions, at a temperature ensuring the growth of the microorganism, preferably at 33 to 40°C, until substantial amounts of the antibiotics are accumulated. The antibiotics are isolated according to methods known per se. 5
- The trace elements can either be present in the form of impurities in other nutritive medium ingredients or can be added separately to the medium. 5
- The air-flow intensity is regulated according to the composition of the medium, the impeller output and other technical characteristics of the fermentor. It is usually advantageous to provide an oxygen input which ensures that the CO₂ content of the outflowing gas-mixture should not exceed 1.0 percent, and that the reducing sugar content should become completely consumed between the 96th and 110th hour in the medium. 10
- The steam-sterilization of the medium is carried out at 121 to 123°C for 30 to 60 minutes. The pH of the medium is preferably adjusted to 7.2 to 7.4 prior to sterilization. 10
- In the course of the fermentation no antifoam agent is added as the oils and/or fats of animal and/or plant origin, added as nutrients to the medium, can simultaneously prevent the foaming of the culture. 15
- Cultivation itself may be carried out in shaker flasks, laboratory, pilot-plant or industrial fermentors. 15
- The antibiotic composition of the cultures is assayed by bioautography following thin-layer chromatography on silica gel. 0.1 to 0.5 µg/ml amounts of the antibiotic complex are spotted to the silica gel thin-layer plates (Merck, Darmstadt), and are submitted to chromatography in a system of ethanol-methyl ethyl ketone-25 percent ammonium hydroxide 1:1:1. Following thorough drying, a medium containing *Bacillus subtilis* ATCC 6633 is spread on the surface of the plate, and is incubated at 20
- 37°C for 16 hours. Then the size of the inhibition zones is compared to that of the standards. This procedure is suitable for qualitative and quantitative assay, standard deviation being ± 10 percent. 25
- The major advantages of the process of the invention are the following: 25
- Streptomyces tenebrarius* MNG 204, producing practically a single component, furnishes higher antibiotic levels than the former parent strain. Further selection works which may be performed on this new strain are promising rapid success as this strain might be free to use its entire biosynthetic capacity for the formation of a single antibiotic component. The biological assay of these fermentation broths containing a single antibiotic component is rapid, simple and exact, the isolation of the antibiotic does not require expensive purification procedures. By mixing the cells of two different strains and cultivating them in a mixed culture, fermentation broths containing nebramycin-2 and nebramycin-5' at any desired preset ratio can be produced according to the actual commercial demands and cost situations. Furthermore it is an additional advantage that in the course of this mixed fermentation neither of the strains loses any substantial part of its productivity, and both synthesize an overall higher level of antibiotics. 30
- The following examples are illustrating but not limiting the scope of the invention. 35
- EXAMPLE 1 40
- a.) Production of fermentation broths containing nebramycin-5' in laboratory fermentors 40
- Agar slants, prepared with distilled water and having the following composition
- | | | |
|--|---------|----|
| dextrin | 1.0 % | |
| yeast extract | 0.1 % | |
| casein hydrolysate (10 %) ⁺ | 2.0 % | 45 |
| meat extract | 0.1 % | |
| cobalt dichloride hexahydrate | 0.001 % | |
| agar | 2.5 % | |
| ⁺ enzymatically hydrolyzed | | |
- are inoculated with the spores or vegetative mycelia of *Streptomyces tenebrarius* MNG 204. The pH of the medium is adjusted with an aqueous sodium hydroxide solution to 7.2 prior to sterilization, then it is sterilized at 121°C for 20 minutes. 50
- The cultures are incubated at 37°C for 4 days, then the spores are washed off the surface of the medium with sterile distilled water and the resulting spore suspension is utilized to inoculate 2 × 100

ml inoculum medium, prepared with tap water and sterilized in 500 ml Erlenmeyer flasks. The inoculum has the following composition:

	soymeal	2.0 %	
	casein hydrolysate (10 %) ⁺	3.0 %	
5	ammonium nitrate	0.1 %	5
	ammonium chloride	0.3 %	
	calcium carbonate	0.3 %	
	magnesium sulfate heptahydrate	0.5%	
	1:1 mixture of palm oil and linseed oil	0.5%	
10	glucose (sterilized separately as a 50 % solution)	2.0 %	10

⁺enzymatically hydrolyzed

The pH of the medium is adjusted with an aqueous sodium hydroxide solution to 7.2, and it is sterilized at 121 °C for 20 minutes in a pressurized vessel.

15 The inoculated cultures are cultivated for 14 to 18 hours at 37 °C on a rotary shaker (diameter 2.4 cm, 280 rpm). 15

200 ml of this fully developed inoculum is used to inoculate a laboratory fermentor having 10 litres of working volume, where 5 l of the main fermentation medium, prepared with tap water, sterilized at 121 °C for 45 minutes, and having the following composition:

20	soymeal	3.0 %	20
	casein hydrolysate (10 %) ⁺	5.0 %	
	ammonium nitrate	0.1 %	
	ammonium chloride	0.5 %	
	L-glutamic acid	0.8 %	
25	calcium carbonate	0.5 %	25
	magnesium sulfate heptahydrate	0.5 %	
	cobalt dinitrate hexahydrate	0.001 %	
	1:1 mixture of palm oil and linseed oil	3.0 %	
30	glucose (sterilized separately as a 50% solution)	4.2 %	30

⁺enzymatically hydrolyzed

is placed.

35 The pH of the medium is adjusted with ammonium hydroxide to 7.2 prior to sterilization. The cultivation is carried out at 37 °C, at an impeller speed of 360 rpm and at a sterile air flow of 3 litres/min. 35

In the 144th hour of cultivation 260 mg of nebramycin-5' and 9 mg of nebramycin-4 are assayed in 100 ml of the broth. No nebramycin-2 is detected.

40 Other nutritive media, having a different composition may be used for the main fermentation, too. Thus, proceeding according to a.), except that the following medium is used. 40

soymeal	3.3%
ammonium chloride	0.6%
magnesium sulfate heptahydrate	0.7%
calcium carbonate	0.5%
cobalt dinitrate hexahydrate	0.001%
palm-oil	2.5%
glucose (sterilized separately in a 50 percent solution)*	2.1%

* Depending on the geometry and accessories of the fermentor used for cultivation, factors influencing cell metabolism, glucose concentration may be increased up to 4.0 percent.

similar results are obtained.

In the 144th hour of cultivation 100 ml of the fermentation broth contains beyond traces of nebramycin-4 (3 mg at its highest level) 180 mg of nebramycin-5'. No nebramycin-2 can be detected.

b.) Production of fermentation broths containing nebramycin-5' in 1000 L pilot plant fermentor
For the production of fermentation broths containing large amounts of nebramycin-5', the procedure described in a.) is applied. 100 ml of the inoculum medium is inoculated with the spores or vegetative mycelia of *Streptomyces tenebrarius* MNG 204, cultivated for 14 to 18 hours, then 100 L of a sterile inoculum medium is inoculated with this culture and cultivated at 37°C, an air-flow of 100 L/min. and an agitation of 360 rpm. After 16 hours 1000 L of the main sterilized fermentation medium (composition see in a.)) is inoculated in a stainless steel fermentor with 50 L of this inoculum. The main fermentation is carried out at 37°C, an air-flow of 500 L/min. and an agitation of 200 rpm. The changes in antibiotic content are represented in Table 2.

TABLE 2

Duration of the fermentation hours	Nebramycin component ($\mu\text{g/ml}$)		
	2	4	5'
24	0	0	50
48	0	0	400
72	0	5	1200
96	0	30	1740
120	0	40	2320
144	0	60	2650

c.) Decarboxylation of nebramycin-5' and isolation of tobramycin
22 g of sodium hydroxide are added to 3.0 L of a fermentation broth containing 2600 $\mu\text{g/ml}$ of nebramycin-5' and 90 $\mu\text{g/ml}$ of nebramycin-4, the broth is heated at constant stirring to 90 to 100°C and kept at this temperature for 25 minutes. When the reaction is concluded, the mixture is cooled to room temperature, and its pH adjusted to 2.0 with a 50 percent aqueous sulfuric acid solution. To this acidic fermentation broth 6 g of oxalic acid is added at constant stirring, its pH is subsequently adjusted to 7.0, the mixture is stirred for a further 30 minutes, filtered, and the mycelium washed twice with 600 ml of water. The filtrate and washings are combined, and the resulting calcium-ion-free solution is submitted to ion-exchange chromatography on a column prepared from 200 ml of Amberlite CG—50 (NH_4^+) (Rohm and Haas Co., Philadelphia, USA). The column is washed with 400 ml of deionized water and eluted first with 1000 ml of a 0.05 N, then with 2000 ml of a 0.1 N and finally with 3000 ml of a 0.15 N solution of ammonium hydroxide. In the course of the elution fractions of 250 ml are collected.

Fractions 13 to 18, containing tobramycin, are combined, the pH of the solution is adjusted with an aqueous 50 percent sulfuric acid solution to 7.0, and submitted to ion-exchange chromatography on 120 ml of Amberlite CG—50 (NH_4^+). The column is washed with 200 ml of deionized water and the antibiotic is eluted first with 1000 ml of a 0.05 N, then with 2000 ml of a 0.1 N and 2000 ml of a 0.15 N, and finally with 2000 ml of a 0.2 N ammonium hydroxide solution. Fractions of 60 ml are collected. Fractions 45 to 56 are combined and evaporated at reduced pressure. The residue, dried over phosphorous pentoxide, yields a product containing 0.7 g (70 percent) of kanamycin B, 12 percent of component N—13 (6'-N-carbamoyl-tobramycin), 10 percent of an unknown component and 8 percent of tobramycin. Evaporating fractions 57 to 58, a residue containing 0.2 g (60 percent) of tobramycin, nearly 40 percent of kanamycin B and 0.1 to 0.2 percent of component N—13 is obtained. The distillation of fractions 59 to 69 yields 5.7 g of tobramycin containing 0.1 to 0.15 percent of component N—13. Recrystallizing this crude tobramycin from aqueous 96 percent ethanol, 4.2 g of pure tobramycin is obtained.

EXAMPLE 2

- 15 Production of fermentation broths containing nebramycin-2 and nebramycin-5' at a preset ratio 15
- Applying the procedure of a.) in Example 1, ten 100 ml portions of an inoculum medium described therein are inoculated with a spore suspension of *Streptomyces tenebrarius* MNG 204, and ten 100 ml portions of the same inoculum medium are inoculated with the spore suspension of *Streptomyces tenebrarius* MNG 169. The microorganisms are cultivated according to a.) in Example 1, then 100 ml portions of a medium sterilized in 500 ml Erlenmeyer flasks, and 5 L portions of a main fermentation medium sterilized in a 10 L fermentor and having a composition described in a.) in Example 1, are inoculated with the two strains either separately, or following their mixing at a ratio set up below.
- 20 20
- Cultivation is continued for 144 hours at fermentation conditions described in Example 1. Then the antibiotic component content is assayed by thin-layer chromatography, subsequent bioautography, and comparison with a standard. The results are summarized in Table 3.
- 25 25

TABLE 3

Cultivation	Microorganism 1 (ml)		Microorganism 2 (ml)		Nebramycin component ($\mu\text{g/ml}$)		
					2	4	5'
Shaken flask							
1	MNG 169	5 ml			5040	180	1310
2	MNG 204	5 ml			0	80	2150
3	MNG 169	4.5 ml	MNG 204	0.5 ml	5640	175	1280
4		4.0 ml		1.0 ml	5140	195	1370
5		3.5 ml		1.5 ml	4740	180	1610
6		3.0 ml		2.5 ml	3140	240	1690
7		2.5 ml		2.5 ml	2850	170	1840
8		2.0 ml		3.0 ml	2310	190	1880
9		1.5 ml		3.5 ml	1320	240	2430
10		1.0 ml		4.0 ml	470	120	2340
11		0.5 ml		4.5 ml	150	95	2340
Fermentor							
I	MNG 169	200 ml			3900	210	870
II	MNG 204	200 ml			0	90	2300
III	MNG 169	175 ml	MNG 204	25 ml	4380	240	1130
IV		150 ml		50 ml	3050	340	1530
V		125 ml		75 ml	2450	220	1640
VI		100 ml		100 ml	2320	420	2150
VII		75 ml		125 ml	1420	300	2470
VIII		50 ml		150 ml	980	160	2540
IX		25 ml		175 ml	280	140	2310

The data of Table 3 clearly demonstrate that fermentation broths containing nebramycin-2 and nebramycin-5' at any desired preset ratio can be produced by applying *Streptomyces tenebrarius* MNG 204 and *Streptomyces tenebrarius* MNG 169. The antibiotics can be isolated from the fermentation broths according to Hungarian Patent No. 174,315.

The bioautography of broths obtained in fermentors I to IX is represented in Figure 1.

CLAIMS

1. A process for preparing fermentation broths containing either practically solely nebramycin-5' or a mixture of nebramycin-2 and nebramycin-5' of any desired preset ratio and nebramycin-4, in which

a) for preparing fermentation broths containing practically solely nebramycin-5' the MNG 204 strain of *Streptomyces tenebrarius*, or

5 b) for preparing fermentation broths which contain nebramycin-2 and nebramycin-5' at any desired preset ratio the spores or vegetative mycelia of any nebramycin complex producing *Streptomyces tenebrarius*, preferably the MNG 169 strain (deposited on December 28, 1980 at the National Collection of Microorganisms, Budapest), and those of any *Streptomyces tenebrarius*, producing practically solely nebramycin-5', preferably the MNG 204 strain (deposited on September 2, 1981 at the National Collection of Microorganisms, Budapest), are mixed at the required ratio, and
10 oils and/or fats of animal and/or plant origin in a submerged, aerated culture, at 33 to 40°C, preferably at 37°C. 10

2. A process according to claim 1, wherein starch, glycerol, fructose, preferably glucose, are employed as organic carbon source, and nutmeal, corn-steep liquor, peptone, yeast extract, preferably soymeal, or casein, amino acids, preferably glutamic acid as organic nitrogen source.
15 3. A process as claimed in claim 1 or 2, substantially as hereinbefore described in Examples 1 and 2. 15

4. A fermentation broth containing nebramycin-5' or a mixture of nebramycin-2 and nebramycin-5' at any desired preset ratio and nebramycin-4, whenever prepared by a process as claimed in any one of claims 1 to 3.
20 5. Nebramycin-5' or a mixture of nebramycin-2 and nebramycin-5' of any desired preset ratio and nebramycin-4, whenever prepared by one of claims 1 to 3. 20

6. A process as claimed in any one of claims 1 to 3, wherein the nebramycin-5' is hydrolysed to give tobramycin either before or after isolation from the broth.

7. Tobramycin whenever prepared by a process as claimed in claim 6.